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# Certificate

The annex attached to this certificate is a copy of the following application filed with our office.

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| Application Date: | March 24, 2004 |
| Application Number: | 200410023827.0 |
| Kind of Application | invention |
| Tile of Invention: | Algin Oligosaccharides and the Derivatives thereof as well as the Manufacture and the Use of the Same |
| Applicant: | OCEAN UNIVERSITY OF CHINA |
| Inventor(s) or Designer(s): | GENG, Meiyu; XIN, Xianliang; YANG, Zhao; GUAN, Huashi; SUN, Guangqian; HU, Jinfeng; FAN, Yin |

The Commissioner of the
State Intelligence Property
Office of the People's
Republic of China

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(seal)

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English translation of CN 200410023827.0

## Claims

1. Use of alginate oligosaccharide in the manufacture of an anti-Alzheimer's disease drug.
2. Use of alginate oligosaccharide in the manufacture of an anti-diabetes drug.
3. The use according to claim 1 or 2, wherein the said mannuronic acid oligosaccharide with reduced terminal in position 1 is carboxyl radical, the guluronic acid oligosaccharide with reduced terminal in position 1 is carboxyl radical and the mannuroguluronate oligosaccharide with reduced terminal in position 1 is carboxyl radical.

English translation of CN 200410023827.0

## Description

### The use of the alginate oligosaccharide against Alzheimer disease and diabete

**Technical Field**

The present invention relates to an alginate oligosaccharide and its derivatives, the preparation thereof, and the uses of the same in the treatment of Alzheimer's disease (AD) and diabetes.

**Background Art**

AD and diabetes are currently common and frequently-occurring diseases which seriously endanger the health of human beings. Particularly, their incidence is increasing with the growth of the population of the old. So the prophylaxis and treatment of these diseases become more and more critical.

Current preventive and curative drugs for AD are unlikely to revolutionize the treatment of AD due to their limitation of the mere symptomatic relief or severe adverse effects. The drugs commonly used for diabetes are mainly insulin and other orally hypoglycemic drugs, most of which are disadvantageous in inconveniency for use and toxicity. Particularly, there are actually no effective drugs for type 2 diabetes.

**Disclosure of Invention**

The destination of the present invention provides an alginate oligosaccharide agonist AD and diabetes in order to retrieve the shortage of present technology.

The alginate oligosaccharide is used to manufacture the drug against Alzheimer's disease.

The alginate oligosaccharide is used to manufacture the drug against diabetes.

The present invention used to prepare the drug against Alzheimer's diseases and diabetes which can be produced in large scale, and the price of which is low.

**Description of drawings and**

Figure 1 shows the mannuronic acid oligosaccharide with reduced terminal in position 1 of carboxyl radical shortened the escape latency of AD animal .

Figure 2 shows the mannuronic acid oligosaccharide with reduced terminal in position 1 of carboxyl radical shortened the swimming distance of AD mice.

Figure 3 shows the mannuronic acid oligosaccharide with reduced terminal in position 1 of carboxyl radical shortened the first time arriving the original plate of AD mice .

Figure 4 shows the mannuronic acid oligosaccharide with reduced terminal in position 1 of carboxyl radical reduced the numbers crossing the original plate of AD mice.

Figure 5 shows the mannuronic acid oligosaccharide with reduced terminal in position 1 of carboxyl radical protected the B cell of the pancreatic island damaged by amylin.

**Embodiments for Carrying Out the Invention**

The present applicant has disclosed three processes for preparing carboxyl algin oligosaccharides having a reducing terminal at position 1 and the algin oligosaccharides thus

produced are a mannuronic acid oligosaccharide having a carboxyl radical at position 1 of the reducing terminal, a guluronic acid oligosaccharide having a carboxyl radical at position 1 of the reducing terminal, and a mannuroguluronate oligosaccharide having a carboxyl radical at position 1 of the reducing terminal, in Chinese patent applications No. 03138966.X, 03138976.7, 03138967.8, respectively. These oligosaccharides can all be produced in large scale and the price of them are cheap. Further tests revealed that these oligosaccharides have the inhibitory effects on Alzheimer's disease and diabete.

**1. Evaluation of the alginate oligosaccharide on Alzheimer's disease (AD)**

With the memory-impaired rats induce by scopolamine peritoneal injection, we evaluated the effects of the present drug on the learning and memory function and relative values of AD rats. The results showed that the mannuronic acid oligosaccharide with reduced terminal in position 1 of carboxyl radical, the guluronic acid oligosaccharide with reduced terminal in position 1 of carboxyl radical and the mannuroguluronate oligosaccharide with reduced terminal in position 1 of carboxyl radical all could improve the learning and memory function significantly, increase the activity of SOD, GSH-PX, ATPase, decrease the content of MDA of cortex and hippocampus in AD rats. These indicated that the mannuronic acid oligosaccharide with reduced terminal in position 1 of carboxyl radical, the guluronic acid oligosaccharide with reduced terminal in position 1 of carboxyl radical and the mannuroguluronate oligosaccharide with reduced terminal in position 1 of carboxyl radical all have the therapeutic effects on AD.

We take a sample of the mannuronic acid oligosaccharide with reduced terminal in position 1 of carboxyl radical to demonstrate their effects.

**Meterials and methods**

1. Drugs and reagents

Ha-bo-yin (HBY) were from HeNan Bamboo Forest Pharmaceuticals company. Scopolamine Hydrobromide Injection (Batch No 020601) were from Shanghai Harvest Pharmaceutical Coperation. The kits of SOD, MDA, GSH-PX and ATPase were all from Nanjing Jiancheng Biological Engineering Institute.

2. Instrument

Morris water maze were designed and manufactured by Institute of Materia Medica, Chinese Academy of Medical Sciences.

3. Animals

Male Wistar rats, weighing 220~250g, provided by Experimental Animal Center of Shandong University.

**Methods**

1. Drug administration

60 rats were randomly divided into 6 groups: control group, model group, the mannuronic acid oligosaccharide groups (40, 120, 360mg/kg) and positive HBY group(40μg/kg). Each group consisted of 10 rats. Except for control and model groups (Sodium Chloride ig.), other groups were administrated corresponding drugs, and successive administration were for 37 days.

2. Morris water maze behavior test

(1) Local navigation test

Local navigation test began from the 31 day, and continued for 5 days. Scopolamine (0.75mg/kg/d) was intraperitoneal injected before 30 minutes in each test. Rats were put into water from 2 stabile positions. The lantency and the swimming trails were recorded within 2 minutes.

(2) Probe exploring test

One day after local navigation test, removing the platform, rats were put into water in one of the stabile position. The swimmig time in the platform quadrant were recorded within 2 minutes.

3. Biochemical indicator test

After morris water maze behavior examination, rats were execute by decapitation, hippocampus and cortex were separated on the ice immediately, stored in -80℃after quick freeze for 1 hour in liquid nitrogen. The cortex and hippocampus were made into 10% and 5% homogenate diluted by sodium chloride, which were centrifugal separated in 3600 r/m. The supernatant liquid were used to test the activity of SOD, MDA, GSH-PX and $Na^+K^+$-ATPase (kits from Nanjing Jiancheng Biological Engineering Institute).

**Statistics**

Statistics and analysis were proceeded by SPSS statistical software and results were demonstrated as "M±SE", compared by ANOVA and t-test.

**Results**

1. Behaviour test

(1) Results of local navigation test

From figure 1 and figure 2, we can see that with the train time prolonged, the escape latency and swimming distance of the rats are reduced gradually. From the 2nd day, compared to control group, the escape latency and swimming distance of rats are significantly prolonged in model group($p<0.01$); in the 5th day, compared to model group, the latency and swimming distance of rats are significantly reduced in the oligosaccharide group and HBY group, indicating that the oligosaccharide obviously improves learning and memory activity of AD rats.

(2) Results of probing test

Figure 3, 4 showed that after removing the platform, compared with the control group, the swimming time staying at the phase of original plate of model group is significantly reduced ($p<0.01$), the number crossing the phase of original plate are significantly elevated. However, the swimming time staying at the phase of original plate are significantly prolonged of the oligosaccharide and HBY group ($p<0.05$, $p<0.01$), indicating that the oligosaccharide obviously improves learning and memory activity of AD rats.

2. The results of biochemical parameters

(1) The effects of the mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical on the ATPase activity of cotex and hippocampus in scopolamine induced AD rats

Compared with scopolamine group, the oligosaccharide improves the $Na^+K^+$-ATPase activity of cortex and hippocampus in scopolamine induced AD rats ($p<0.05$, $p<0.01$, $p<0.001$, table 1).

Table 1.Effects of the mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical on the ATPase activity of cotex and hippocampus in scopolamine induced AD rats ($\bar{x}\pm SE$, n=10)

| group | dose (mg/kg) | $Na^+K^+$-ATPase activity (umol pi / mg.prot. hr) | |
|---|---|---|---|
| | | hippocampus | cortex |
| control | — | 8.67±0.40 | 6.72±0.13 |
| model | — | $4.90\pm0.21^{\#\#\#}$ | $4.12\pm0.44^{\#\#\#}$ |
| oligosaccharide | 40 | $8.06\pm0.37^{***}$ | $6.62\pm0.25^{***}$ |
| | 120 | $6.30\pm0.57^{**}$ | $6.97\pm0.45^{***}$ |
| | 360 | $8.35\pm0.54^{***}$ | $5.71\pm0.33^{***}$ |
| HBY | 0.04 | $7.04\pm0.45^{***}$ | $6.50\pm0.21^{***}$ |

$^{\#\#\#}p<0.001$, compared with control group; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with model group

(2) Effects of the mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical on the SOD activity of cortex and hippocampus in scopolamine induced AD rats

The results showed in table 2, the mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical improves the SOD activity both in cortex and hippocampus ($p<0.05$, $p<0.01$, $p<0.001$).

Table 2. Effects of mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical on the CuZn-SOD activity of cortex and hippocampus in scopolamine induced AD rats ($\bar{x}\pm SE$, n=10)

| group | dose (mg/kg) | CuZn-SOD activity (NU/mg.prot.) | |
|---|---|---|---|
| | | hippocampus | contex |
| control | — | 127.83±7.34 | 125.71±8.32 |
| model | — | $118.55\pm4.95^{\#\#}$ | $115.54\pm8.13^{\#\#}$ |
| oligosaccharide | 40 | $124.67\pm7.25^{*}$ | $130.47\pm8.94^{***}$ |
| | 120 | 119.54±7.42 | $148.14\pm9.74^{***}$ |
| | 360 | $126.03\pm6.80^{**}$ | $139.20\pm7.79^{***}$ |
| HBY | 0.04 | 128.80±8.33 | 114.10±5.97 |

$^{\#\#\#}p<0.001$, compared with control group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with model group

(3) Effects of mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical on the GSH-PX activity of cortex and hippocampus in scopolamine induced

AD rats

The results show in table 3, the mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical improves the GSH-PX activity both in cortex and hippocampus($p<0.05$, $p<0.01$).

Table 3. Effects of mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical on the GSH-PX activity of cortex and hippocampus in scopolamine induced AD rats ($\bar{x}\pm SE$, n=10)

| group | dose (mg/kg) | GSH-PX activity (U/mg.prot.) | |
|---|---|---|---|
| | | hippocampus | contex |
| control | — | 6.13±0.44 | 8.33±0.18 |
| model | — | 5.63±0.38 | $7.18\pm0.29^{\#\#}$ |
| oligosaccharide | 40 | 6.37±0.33 | $9.54\pm0.61^{**}$ |
| | 120 | 5.72±0.30 | $8.97\pm0.61^{*}$ |
| | 360 | 6.07±0.38 | $8.45\pm0.48^{*}$ |
| HBY | 0.04 | 6.30±0.29 | $8.20\pm0.40^{*}$ |

$^{\#\#\#}p<0.001$, compared with control group, $^{**}P<0.01$, $^{***}P<0.001$, compared with model group

(4) Effects of mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical on the MDA content of cortex and hippocampus in scopolamine induced AD rats

The results show in table 4, the mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical decreases the MDA content both in cortex and hippocampus ($p<0.05$, $p<0.01$)

Table 4. Effects of mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical on the MDA content of cortex and hippocampus in scopolamine induced AD rats ($\bar{x}\pm SE$, n=10)

| group | dose (mg/kg) | MDA (nmol/ mg prot.) | |
|---|---|---|---|
| | | hippocampus | contex |
| control | — | 2.35±0.21 | 3.25±0.11 |
| model | — | $4.46\pm0.21^{\#\#\#}$ | $4.16\pm0.12^{\#\#\#}$ |
| oligosaccharide | 40 | $2.65\pm0.14^{***}$ | $3.24\pm0.16^{***}$ |
| | 120 | $1.83\pm0.09^{***}$ | $3.64\pm0.14^{**}$ |
| | 360 | $1.17\pm0.05^{***}$ | $2.69\pm0.16^{***}$ |
| HBY | 0.04 | $2.48\pm0.18^{***}$ | 4.36±0.11 |

$^{\#\#\#}p<0.001$, compared with control group, $^{**}P<0.01$, $^{***}P<0.001$, compared with model group

**2. Evaluation of the alginate oligosaccharide on diabete**

With pancreatic beta-cells NIT cell line impaired by amylin(IAPP), the protection of the alginate oligosaccharide was observed.The results showed that the alginate oligosaccharide could increase the survived cells impaired by amylin in a dose-dependent manner. These data indicated that oligosaccharide has protective effects on amylin-impaired diabetic pancreatic beta-cells.

With the diabetic mice induced by streptozotocin (STZ), the therapeutical effect of the alginate oligosaccharide on the diabetic mice was observed. The blood glucose concentration in the alginate oligosaccharide group is significantly lower than that of the model group, indicating that oligosaccharide has therapeutic effects on STZ-induced diabetic mice.

We take a sample of the mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical to demonstrate the materials, methods and results.

**Meterials and methods**

1. Drugs and reagents

amylin（IAPP）and streptozotocin were bought from SIGMA。

2. Animal

NIH mice, male, weighing 18-22 g, provided by Animal Center of Chinese Academy of Medical Sciences

**Methods**

The human pancreatic beta-cells cell line NIT is cultured with DMEM medium containing 10% FBS. The cells are seeded into 96-well plates in density of $1\times10^4$ cells/well. The day after plating, they are pretreated with varying concentrations of oligosaccharide(final concentration of 0, 10, 50, 100 μg/ml) for 24 h, followed by the addition of aged amylin with a final concentration of 30 μM. After 48 h at 37°C, the survival of the cells is measured by MTT method.

The results showed that oligosaccharide could increase the survived cells impaired by amylin in a dose-dependent manner. The data suggested that the mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical has protective effects on pancreatic beta cells impaired with amylin(Fig.5)

Sixty male NIH mice (weighed 18-22g) are randomly divided to control, model, 50,150,450 mg/kg oligosaccharide-treated and 5mg/kg dimethyldiguanide-treated groups. The mice are injected intraperitoneally with 150mg/kg STZ except control group at the 1st day. Then the mice are given accordingly drugs consecutively for 10 days and blood were taken on the 11th day. The blood is taken to measure the glucose concentration. The concentration in each oligosaccharide-treated group is significantly lower than that in the model group, indicating that oligosaccharide has therapeutic effects on STZ-induced diabetic mice (table 5).

Table 5. Effects of oligosaccharide on the blood glucose concentration of diabetic mice induced by STZ ($\bar{x}\pm$SD)

| Group | Dose (mg/kg) | Number of mice | Blood glucose concentration (mg/dL) |
|---|---|---|---|
| Control | — | 10 | 150.6±36.8 |
| Model | — | 10 | 312.4±89.2$^{\#\#\#}$ |
| 6-mer | 50 | 10 | 219.4±67.8$^{*}$ |
| | 150 | 10 | 179.6±69.8$^{**}$ |
| | 450 | 10 | 162.5±3$^{**}$ |
| Dimethyldiguanide | 5 | 10 | 201.6±58.9$^{**}$ |

$^{\#\#\#}$ P < 0.001 vs control; * P < 0.05, **p<0.01 vs model

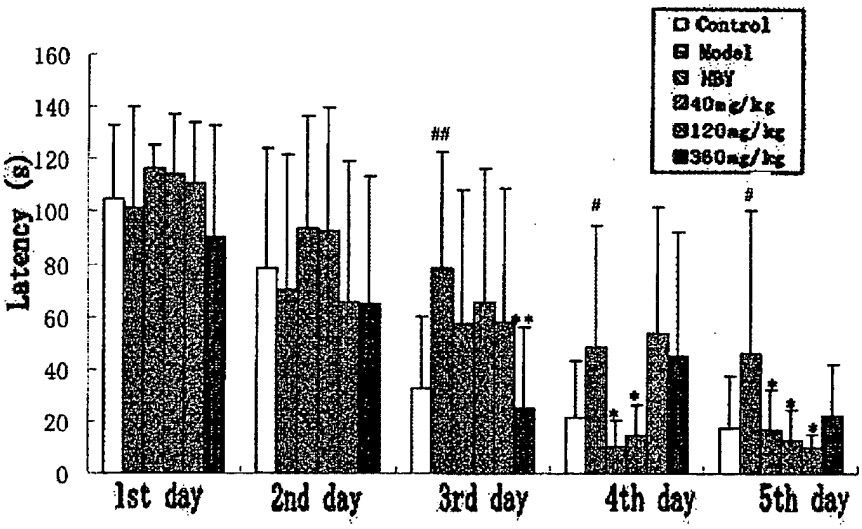


Figure 1.Effects of mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical on the escape latency of AD rats induced by scopolamine.
$^{\#}$ P < 0.05, $^{\#\#}$ P < 0.01 vs control; * P < 0.05, vs model

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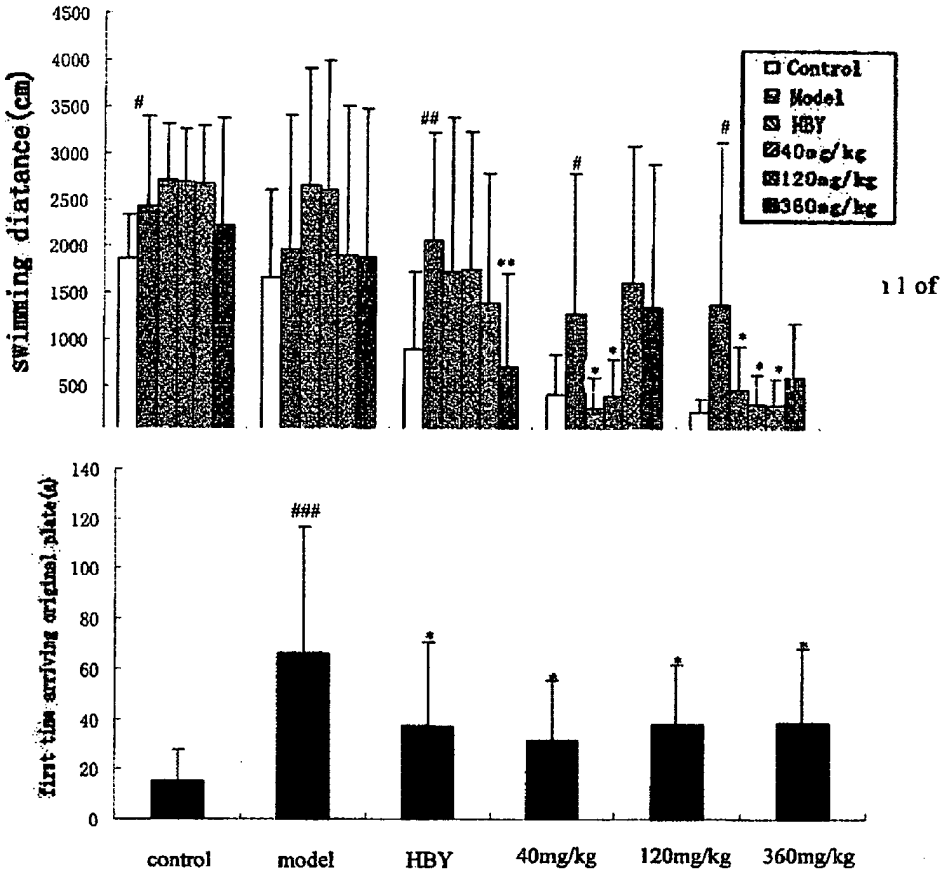


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Figure 3. Effects of mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical on the first time arriving original plate of AD rats induced by scopolamine. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs control; * $P < 0.05$, ** $P < 0.01$vs model

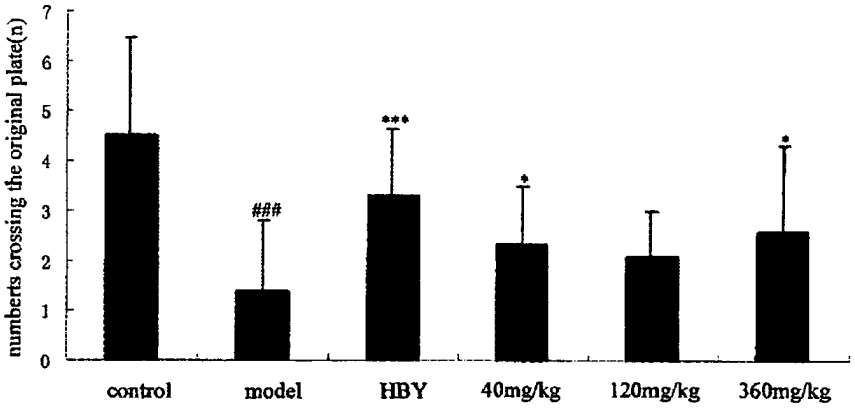


Figure 4. Effects of mannuronic acid oligosaccharide with reduced terminal position 1 of carboxyl radical on numbers crossing the original plate of AD rats induced by scopolamine.
###P < 0.001 vs control; * P < 0.05, *** P < 0.001vs model

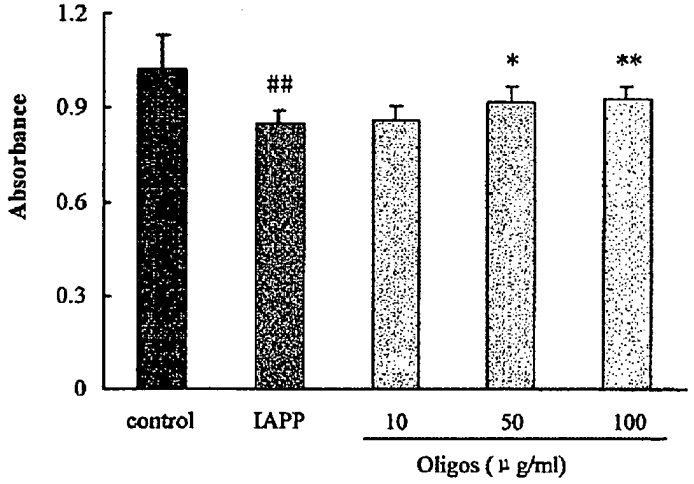


Figure 5. Protective effects of mannuronic acid oligosaccharide with reduced terminalposition 1 of carboxyl radical on the damage of NIT cell induec by IAPP
##P < 0.01 vs control; * P < 0.05, ** P < 0.01vs model

# CERTIFICATE OF VERIFICATION

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state that the attached document is a faithful and complete translation to the best of my knowledge of Chinese Patent Application No. CN 200410023827.0.

Dated this 4th day of August 2009

Signature of Translator: Zheng Fan ;